

REMARKS

Claims 11-13, 16, 20-22, 24, 33, 34, 38-40, and 47-55 are pending.

This is in response to the Final Office Action dated December 23, 2009. Applicants respectfully request reconsideration of the rejection in light of the following remarks.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 11-13, 16, 20-22, 24, 33, 34, 38-40, and 47-55 remain rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to comply with the enablement requirement. Applicants strongly disagree and traverse the rejection.

Based on a comparison of the fatty acid content between the transgenic lines and the C-24 wild type control, the Office Action concludes that the transformed lines do not show an increase in fatty acids (Final Office Action, page 3). Applicants respectfully submit that the comparison was made with the wrong control and that when the correct comparison is made, the transgenic lines comprising the gene of interest do indeed show increased mean total fatty acid content when compared to the Col-2 empty vector control as depicted in Table 16.

Applicants direct the Examiner's attention to Example 15 at page 65 of the specification which explains the controls used in the experiments. Specifically, the specification recites:

"C-24 (an Arabidopsis ecotype found to accumulate high amounts of total fatty acids in seeds) . . . The controls indicated in the tables below have been grown side by side with the transgenic lines." (Specification, page 65, lines 7-10).

Two controls were used in the experiments, *i.e.* the "Col-2 empty vector control" and the "C-24 wild-type control." The "C-24 wild type control" is a high oil producing line that was not transformed (non-transgenic line) used to show that high oil production under the agronomic conditions of the experiments is possible. The "Col-2 empty vector control" is transgenic but does not express the gene of interest.

The experimental data shown in Table 16 at page 68, for example of the "Pk239-20 transgenic seeds" line expressing SEQ ID NO: 23 compared to the correct control, *i.e.* "Col-2

empty vector control,” clearly show that the mean fatty acid content is increased in the transgenic seed comprising SEQ ID NO: 23.

Furthermore, O'Neill *et al.* confirm that C-24 is a high oil producing line (41% seed oil) compared with the Col-2 line (only 29% seed oil). See page 1079 of O'Neill *et al.*, 2003, Natural variation of seed oil composition in *Arabidopsis thaliana*. *Phytochemistry* 64:1077-1090 (a copy of which is enclosed for the Examiner's convenience).

The Final Office Action at page 3 alleges that Applicants had argued that Dormann shows no increase in fatty acids in transformed plants relative to wild type plants. Applicants arguments have been mischaracterized. The Examiner is directed to page 9 lines 18-19 of the Amendment and Reply under 37 CFR § 1.111 dated September 1, 2009. Contrary to the Examiner's assertion, Applicants rather stated that “Dormann is silent on the effect of the *dgd* mutation or DGD1 on overall fatty acid levels or fatty acid levels in seed.” (emphasis added). Dormann discloses that there are two DGD genes in *Arabidopsis* and strong regulation between the two pathways for galactosyl lipid biosynthesis. Dormann does not teach or suggest the effect of expression of one DGD alone, but rather a disruption in the pathway preventing further biosynthesis of the precursor. (Dormann, page 2182).

Because the enablement rejection is premised on an erroneous interpretation of the data of Table 16, Applicants submit that when the correct comparison is made, the arguments for lack of enablement are no longer applicable. The specification clearly demonstrates that the mean fatty acid content is increased in transgenic seed comprising the gene of interest relative to seed that do not comprise the gene of interest. *In re Angstadt*, 537 F.2d 498 (CCPA 1976) (There has never been a requirement that every species encompassed by a claim must be disclosed or exemplified in a working example.).

In light of the comments above, Applicant's attorney respectfully reminds the Examiner that the representations in the specification as to the manner of making and using the claimed invention must be taken as in compliance with the first paragraph of 35 U.S.C. §112, unless there is objective evidence or scientifically based reasoning inconsistent with the specification. See *In re Marzocchi and Horton*, 169 U.S.P.Q. 367 (C.C.P.A. 1971). “It is the Patent Office's

burden to present evidence that there is some reason to dispute the enablement provided in the specification. Unsupported speculation or conjecture on that the invention "might not work" will not support a rejection based on 35 U.S.C. §112, first paragraph." *Id.*

Given the correct interpretation of the data in the Examples, the Examiner has not presented the evidence necessary to dispute the enablement provided in the instant specification. Accordingly, the Patent Office has not met its burden and reconsideration and withdrawal of the enablement rejections is requested.

CONCLUSION

For at least the above reasons, Applicants respectfully request withdrawal of the rejections and allowance of the claims. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number given below.

Accompanying this response is a Notice of Appeal and a petition for a two-month extension of time to and including May 24, 2010 pursuant to 37 CFR § 1.7(a) to respond to the Office Action mailed December 23, 2010 with the required fees paid by credit card. No further fee is believed due with this response. However, if a fee is due, the Director is authorized to charge our Deposit Account No. 03-2775, under Order No. 12810-00379-US from which the undersigned is authorized to draw.

Respectfully submitted,

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Natural variation for seed oil composition in *Arabidopsis thaliana*

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Abstract

The biochemical pathways involved in the biosynthesis and accumulation of storage lipids in seeds have been extensively studied. However, the regulatory mechanisms of those pathways, their environmental interactions and the ecological implications of variation are poorly understood. We have initiated a new approach: the analysis of natural variation in *Arabidopsis thaliana*. Three hundred and sixty accessions were surveyed for content of oil, very long chain fatty acids (VLCFAs) and polyunsaturated fatty acids (PUFAs) in their seeds. The results revealed extensive natural variation. A core set of accessions, the seeds of which reproducibly contain extreme amounts of oil, VLCFAs and PUFAs have been identified. Reproducible oil content ranged from 34.6 to 46.0% of seed dry weight. VLCFA content ranged from 13.0 to 21.2% of total fatty acids. PUFA content, ranged from 53.3 to 66.1% of total fatty acids. Interactions were also identified for PUFA and VLCFA content of seeds with vernalisation of plants. Mapping of the regions of the genome involved in controlling the traits was conducted in an F₂ population and indicated that natural variation at the loci *FAE1* and *FAD3* might be involved in the regulation of VLCFA and PUFA content, respectively. A set of accessions, which capture a broad range of the natural variation for these traits available in *A. thaliana*, has been selected to form a core set which can be used to further dissect the genetics of the regulation of seed lipid traits and to identify the genes involved.

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Keywords: *Arabidopsis thaliana*; Natural variation; Seed lipid traits; *FAE1*; *FAD3*

1. Introduction

Arabidopsis thaliana is an oilseed species that has been developed as a model system and used for the genetic analysis of a wide range of developmental and physiological processes, including lipid biosynthesis (Ohlrogge and Browse, 1995). Its complete genome sequence is available (Arabidopsis Genomic Initiative, 2000) as is an array of functional genomics resources. Seed physiology and development in *A. thaliana* is similar to that of the commercially important *Brassica* oilseed crops, to which *A. thaliana* is closely related. The genes of *B. napus* and *A. thaliana* typically show ca. 85% nucleotide identity in coding regions (Cavell et al., 1998). Consequently, *A. thaliana* has become an important model for *Brassica* oilseed crops. Oil is stored largely as triacylglycerols (TAGs), which are synthesised by the enzymes of the Kennedy pathway, by the sequential

acylation of a glycerol backbone. The fatty acid composition of TAG can vary both within and between species. To begin to dissect the genetics of this system in *A. thaliana*, large-scale chemical mutagenesis experiments were carried out. A series of mutants were generated, in which genes in the fatty acid synthesis pathway had been inactivated (James and Dooner, 1990; Lemieux et al., 1990) and subsequently used for the identification of the genes involved. These include mutants with reduced fatty acid desaturation and fatty acid elongation, such as those at loci *FAD2*, *FAD3* and *FAE1* (Okuley et al., 1994; Arondel et al., 1992; James et al., 1995) and mutations at loci such as *TAG1*, which encodes diacylglycerol acyltransferase (the enzyme that catalyses the final acylation of the glycerol backbone and is the only step of the Kennedy pathway that is committed to TAG synthesis), which lowers storage oil content in mature seeds and affects the fatty acid composition of the oil (Katavic et al., 1995; Poirier et al., 1999).

The genetics of storage lipid accumulation has been studied in only a very few accessions of *A. thaliana*.

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However, seed lipid composition may be regarded as an adaptive trait, with fine-tuning potentially conferring an advantage in specific environmental conditions. This would give rise to natural variation that could be used to identify the loci involved in the regulatory mechanisms of the biosynthesis and accumulation of storage lipids, and in particular their environmental interactions. *A. thaliana* has been collected from a wide range of geographical locations throughout the world and the analysis of natural variation is proving highly valuable for understanding the regulation of other adaptive traits. Examples include flowering time, seed and plant size, glucosinolates and epicuticular wax composition (Koorneef et al., 1998; Mitchell-Olds, 1996; Alonso-Blanco et al., 1999; Krannitz et al., 1991; Mithen et al., 1995; Rashotte et al., 1997).

Our first aim was to conduct the first comprehensive study of seed lipid characters in all of the *A. thaliana* accessions available from the *Arabidopsis* resource centres. Previous studies examining these traits did so for a limited numbers of accessions (Pathak et al., 1994; Millar and Kunst, 1999). Our second aim was to identify, for the study of the regulation of seed oil synthesis, a core set of accessions that captures the maximum genetic diversity in a small number of lines. Studying natural variation for these traits will permit the analysis of both allelic variation of the genes known to be involved in the biosynthetic pathways and the identification of other loci involved in the control of the process. Exploiting natural genetic variation is complementary to previous research on mutants, and will provide a more complete understanding of the regulation of seed oil content and fatty acid composition. Our final aim was to identify some of the genetic loci that are candidates for involvement in the control of seed lipid traits.

2. Results

2.1. Measurement of seed lipid traits

Plants representing 360 accessions of *A. thaliana* were grown to maturity in a temperature-controlled glasshouse supplemented with sodium lamps. All plants were vernalised for 6 weeks at the seed stage and grown under 16 h photoperiod conditions to promote early flowering. Logistically it was not possible to achieve this with replica tests of the accessions, due to the requirement to grow the plants with adequate space among accessions to prevent significant cross-pollination. Also, we anticipated that the traits we aimed to measure are environmentally responsive, so the imperative was to grow all plants simultaneously in the same glasshouse. Therefore, the initial survey screen was conducted without replication of accessions. However, four plants

(of the same accession) were grown per pot and the seeds were pooled to reduce the effect of plant-to-plant variation. Comparisons were made between duplicate accessions to gauge the reliability of the data obtained. The "core accessions" were selected to represent maximum genetic diversity for alleles involved in the control of seed lipid traits and were subsequently analysed under similar glasshouse conditions, with 10 replicates.

Seeds were collected from mature, dried-off plants and contaminating material was removed by hand. Aliquots of cleaned seeds weighing ca. 50 mg were analysed for oil content on a dry weight basis using nuclear magnetic resonance spectroscopy. The fatty acid composition of seed lipids was determined by the analysis of fatty acid methyl esters extracted from 50 mg of cleaned seeds and analysed by gas chromatography. Using this system, we were able to unambiguously identify peaks on the chromatograms corresponding to the methyl esters of nine fatty acids. The fatty acid composition of oil in the seeds of accession Columbia (the reference accession, which is typical for fatty acid composition), when grown under standard conditions (i.e. in a glasshouse, without vernalisation treatment) is shown in Table 1. There were three additional minor peaks on chromatographs, which occurred in various proportions in all samples. However, these could not be assigned to known fatty acids.

In order to address the biological significance of the variation of the nine fatty acids measured, we defined ratio measurements to characterise the proportions of VLCFAs and PUFAs. We define the "fatty acid chain length ratio" as the sum of areas under peaks for all 20- and 22-carbon fatty acids divided by the sum of areas under peaks for all 16- and 18-carbon fatty acids. There appears to be very little polyunsaturation of 16- and 20-carbon fatty acids in *A. thaliana*, so we define the "fatty acid desaturation ratio" as the sum of areas under peaks for polyunsaturated 18-carbon fatty acids divided by the sum of areas under peaks for saturated and mono-unsaturated 18-carbon fatty acids. These ratios negate the impact of variation in the unassigned peaks on the

Table 1
Fatty acid composition of seeds from accession Columbia

Name	Symbol	% ^a	S.D.
Palmitic	16:0	9.6	0.11
Stearic	18:0	2.7	0.10
Oleic	18:1	10.8	0.47
Linoleic	18:2	32.8	0.91
Linolenic	18:3	21.2	0.40
Arachidic	20:0	1.2	0.05
9-Eicosenoic	20:1	11.4	0.44
9, 12-Eicosenoic	20:2	1.7	0.08
Erucic	22:1	0.9	0.53

^a Percentage of total integrated peaks on chromatographs; mean of nine plants.

Table 2
Percentage seed oil, chain length and desaturation ratios for the 360 accessions

Accessions	% Oil	Chain length ratios	Desaturation ratios	Accessions	% Oil	Chain length ratios	Desaturation ratios
Aa-0 (N900)	35	0.27	3.2	Rubezhnoe-1	38	0.22	3.9
Abd-0	31	0.24	3.9	Shuhdara	38	0.17	4.3
Ag-0 (N901)	35	0.29	3.1	Su(5)-1	42	0.21	2.6
Cnr-1	32	0.26	3.2	Sorbo	33	0.17	3.3
Col-2	29	0.19	3.2	s96	35	0.24	3.5
Col-3	36	0.2	3.0	Tsu-1	34	0.23	3.0
Col-4	38	0.21	3.0	Wassilewskija	40	0.27	3.2
Col-5(g11)	38	0.22	3.0	Ws-1	38	0.27	3.3
Kondara	35	0.15	4.2	Ws-2	37	0.25	3.4
Cvi-0 (N902)	28	0.2	4.4	Ws-3	39	0.28	3.3
C24	41	0.27	3.2	Wei-0	40	0.24	2.3
Da(1)-12	39	0.25	2.5	Wei-1	39	0.22	2.2
Dijon G	39	0.22	2.2	Aa-0 (N934)	32	0.22	2.7
Dijon M	44	0.24	2.4	Ag-0 (N936)	34	0.25	2.9
Ema-1	38	0.26	3.3	Ak-1	37	0.25	2.7
Enkheim-D	43	0.22	2.4	An-1	37	0.22	3.3
Enkheim-T	38	0.21	3.3	An-2	41	0.26	3.4
Estland	37	0.21	3.3	Ang-0	34	0.26	3.5
Hodja-Obi-Garm	36	0.16	4.3	Ang-1	36	0.27	3.2
H55	36	0.24	3.9	Ba-1	37	0.25	3.3
Je54	34	0.22	2.9	Bay-0	39	0.21	3.1
Kas-1 (N903)	38	0.25	3.6	Beh-1	38	0.25	3.2
Landaberg	32	0.22	2.4	Beh-3	43	0.25	3.5
Ler-1	31	0.21	2.5	Beh-4	43	0.21	2.6
Mh-0 (N904)	41	0.24	2.7	Bd-0	40	0.21	3.1
Ms-0 (N905)	34	0.25	3.8	Be-1	40	0.21	2.7
Nd-1	36	0.23	3.2	Bl-1	41	0.22	2.8
Oy-1	39	0.24	2.7	Bla-1	42	0.23	2.9
Petargof	39	0.24	2.8	Bla-2	42	0.26	2.6
RLD1	38	0.25	2.8	Bla-3	41	0.27	3.2
Rld-2	35	0.24	3.2	Bla-4	37	0.23	3.6
Bla-5	37	0.25	3.0	Bu-23	40	0.22	3.2
Bla-6	38	0.28	3.3	Bu-24	40	0.23	2.8
Bla-10	41	0.27	3.2	Bu-25	40	0.23	3.1
Bla-11	41	0.24	3.3	Bur-0	40	0.2	3.1
Bla-12	37	0.18	3.2	Ca-0	40	0.25	2.7
Bla-14	38	0.21	3.5	Cal-0	37	0.22	3.4
Blh-1	41	0.24	3.0	Can-0	37	0.2	3.4
Blh-2	43	0.21	2.8	Cen-0	41	0.25	3.2
Br-0	40	0.3	3.1	Cha-0	39	0.18	2.7
Bs-1	38	0.24	2.7	Cha-1	38	0.29	3.4
Bs-2	42	0.22	2.0	Chi-0	43	0.21	3.1
Bs-5	39	0.29	2.4	Chi-1	39	0.22	3.2
Bsch-0	38	0.26	2.6	Chi-2	39	0.22	2.4
Bsch-2	38	0.26	3.0	Cit-0	38	0.31	4.2
Bu-0	40	0.26	2.3	Cl-0	39	0.21	2.4
Bu-2	36	0.26	2.5				
Bu-3	37	0.23	3.2	Co-1	43	0.27	2.8
Bu-4	38	0.27	2.5	Co-2	36	0.25	2.6
Bu-5	40	0.27	2.5	Co-3	35	0.27	3.2
Bu-6	37	0.28	3.1	Co-4	38	0.25	3.0
Bu-7	38	0.25	2.8	Col-0	36	0.24	4.3
Bu-8	40	0.29	3.3	Ct-1	44	0.23	2.5
Bu-9	37	0.26	2.8	Cvi-0 (N1096)	31	0.24	4.3
Bu-13	37	0.26	2.7	Du-0	42	0.21	3.3
Bu-14	39	0.22	3.0	Db-0	42	0.22	2.5
Bu-15	37	0.23	3.0	Db-1	41	0.22	2.5
Bu-17	41	0.27	3.3	Db-2	42	0.23	2.6
Bu-18	42	0.21	3.3	Di-0	40	0.24	2.6
Bu-19	41	0.27	3.1	Di-1	39	0.26	3.2

(continued)

Table 2 (continued)

Accessions	% Oil	Chain length ratios	Desaturation ratios	Accessions	% Oil	Chain length ratios	Desaturation ratios
Bu-20	41	0.21	3.1	Dr-0	38	0.22	2.7
Bu-21	42	0.24	2.6	Dra-0	40	0.26	2.4
Bu-22	39	0.21	3.7	Gie-0	38	0.24	3.1
Dra-1	37	0.25	3.5	Goo-0	39	0.24	3.9
Dra-2	37	0.22	2.5	Gr-1	41	0.24	3.8
Edi-0	41	0.23	3.4	Gr-2	41	0.23	2.9
Ei-2	42	0.24	3.8	Gr-5	42	0.24	2.5
Ei-4	40	0.22	3.4	Gr-6	42	0.35	2.9
Ei-5	36	0.2	2.6	Gre-0	41	0.27	3.6
Ei-6	39	0.22	2.7	Gue-0	40	0.24	3.1
Eil-0	42	0.23	2.8	Gue-1	40	0.24	2.5
El-0	43	0.26	3.2	Gy-0	37	0.32	3.8
En-1	42	0.22	2.4	Ha-0	38	0.24	3.0
En-2	39	0.2	3.1	Hi-0	39	0.21	2.6
Ep-0	39	0.24	2.9	Hi-1	38	0.3	3.5
Er-0	41	0.23	2.9	Hi-2	41	0.25	3.2
Fs-0	37	0.23	3.6	Hi-3	39	0.33	3.9
Est-0	39	0.22	3.4	Hn-0	41	0.26	3.3
Est-1	40	0.25	3.2	Is-0	43	0.29	3.0
Et-0	40	0.26	2.8	In-0	42	0.23	2.6
Fe-1	40	0.27	2.8	Is-1	39	0.29	3.9
Fl-1	40	0.27	3.8	Is-2	42	0.24	3.8
Fr-2	43	0.24	3.2	Ita-0	35	0.24	3.7
Fr-3	44	0.25	2.3	Je-0	39	0.27	3.2
Fr-4	40	0.25	3.7	Jl-1	38	0.26	2.9
Fr-5	38	0.24	3.7	Jl-2	40	0.27	2.6
Fr-6	39	0.24	3.0	Jl-3	40	0.28	3.2
Fr-7	35	0.2	2.8	Jl-4	41	0.26	2.5
Ga-0	45	0.33	2.7	Jl-5	40	0.24	2.6
Ga-2	36	0.23	2.9	Jm-0	39	0.25	3.2
Gd-1	42	0.2	2.9	Jm-1	38	0.26	2.3
Ge-0	39	0.25	3.1	Jm-2	38	0.23	2.3
Gc-1	40	0.23	2.6	Kac-0	35	0.24	2.8
Ge-2	36	0.23	2.9	Li-0	38	0.25	3.2
Kas-1 (N1264)	40	0.19	3.4	Li-1	40	0.28	3.0
Kb-0	40	0.26	2.8	Li-2	40	0.27	3.0
Kil-0	42	0.25	2.4	Lm-2	38	0.27	3.0
Kin-0	41	0.26	2.7	Loc-1	39	0.26	2.8
KI-0	41	0.26	2.7	Loc-2	37	0.27	3.0
KI-1	37	0.26	3.7				
KI-2	40	0.30	3.5				
KI-5	41	0.27	2.6	Lu-1	39	0.27	3.0
Kn-0	40	0.29	3.8	Lz-0	37	0.26	2.6
Ko-2	37	0.26	3.6	Ma-0	34	0.25	2.8
Kr-0	37	0.25	2.7	Ma-2	34	0.24	2.9
Kro-0	39	0.23	3.3	Mc-0	45	0.26	2.6
La-0	42	0.21	2.0	Me-0	39	0.21	3.0
La-1	38	0.28	3.5	Mh-0 (N1366)	40	0.23	3.3
Lan-0	38	0.25	2.8	Mh-1	43	0.23	3.1
Lc-0	38	0.22	2.5				
Le-0	41	0.26	3.0	Mnz-0	35	0.25	3.6
Li-1	39	0.25	3.4	MrK-0	38	0.26	2.9
Li-2	34	0.24	2.9	Ms-0 (N1376)	37	0.21	2.6
Li-2-1	39	0.24	3.3	Mi-0	34	0.24	3.3
Li-3	36	0.24	3.1	Mv-0	42	0.27	3.4
Li-3-3	32	0.25	2.8	Mz-0	39	0.25	2.1
Li-5	33	0.23	3.0	Na-1	38	0.28	2.7
Li-5-2	38	0.24	2.9	Nc-1	33	0.25	2.8
Li-5-3	38	0.24	2.6	Nd-0	38	0.24	2.9
				Nic-0	37	0.22	3.0
Li-6-1	38	0.27	3.2	No-0	37	0.25	2.9

(continued)

Table 2 (continued)

Accessions	% Oil	Chain length ratios	Desaturation ratios	Accessions	% Oil	Chain length ratios	Desaturation ratios
Li-7	38	0.27	3.0	Nok-0	38	0.24	2.8
Li-8	40	0.23	3.3	Nok-1	35	0.25	2.4
Li-10	37	0.27	3.2	Nok-2	42	0.27	2.8
Lip-0	40	0.24	2.8	Nok-3	28	0.24	3.1
Np-0	42	0.21	2.8	Po-0	34	0.26	2.6
Nw-0	36	0.23	2.8	Po-1	36	0.24	2.9
Nw-1	41	0.22	2.8	Pog-0	38	0.25	3.3
Nw-2	36	0.2	3.2	Pr-0	37	0.26	2.9
Nw-3	41	0.23	2.4	Pt-0	36	0.26	2.9
Nw-4	35	0.23	3.1	Ra-0	38	0.23	2.9
Ob-0	34	0.21	3.1	Rak-2	37	0.25	2.9
Ob-1	37	0.25	2.8	Rd-0	36	0.25	3.0
Ob-2	39	0.22	2.6	Ri-0	41	0.23	2.8
Ob-3	36	0.26	3.0	Rou-0	34	0.28	2.9
Old-1	36	0.25	2.8	Rsch-0	37	0.25	3.5
Old-2	38	0.26	2.9	Rsch-4	37	0.23	3.6
Ost-0	33	0.27	4.3	Ru-0	36	0.22	2.9
Or-0	32	0.27	4.1	Sah-0	38	0.21	2.9
Ove-0	40	0.25	3.4	Sup-0	38	0.24	3.1
Oy-0	36	0.26	2.8	Sav-0	40	0.24	2.6
Pa-1	40	0.24	2.7	Se-0	35	0.25	3.0
Pa-2	38	0.20	2.9	Sei-0	34	0.22	4.0
Pa-3	35	0.26	3.3	Sf-1	37	0.26	3.4
Per-1	34	0.23	3.4	Sf-2	40	0.25	2.9
Per-2	36	0.22	3.4	Sg-1	32	0.23	2.9
Per-3	35	0.22	2.8	Sg-2	31	0.23	3.2
Pf-0	35	0.25	3.3	Sh-0	39	0.22	3.2
Pj-0	34	0.25	2.8	Si-0	36	0.23	3.8
Pl-2	39	0.24	3.1	Sp-0	35	0.25	3.2
Pla-0	33	0.25	2.9	St-0	32	0.33	1.8
Pla-1	34	0.24	2.9	Stc-0	35	0.26	3.9
Pla-2	38	0.25	3.0	Stw-0	40	0.23	3.3
Pla-3	37	0.27	3.3	Su-0	37	0.22	3.0
Pla-4	36	0.23	2.9	Ta-0	35	0.21	2.9
Pn-0	37	0.21	2.8	Te-0	39	0.25	3.5
Ts-1	36	0.25	3.0	X-0	39	0.25	3.1
Ts-2	36	0.25	2.9	XX-0	37	0.23	3.3
Ts-3	38	0.25	3.1	XXX-0	33	0.19	3.2
Ts-5	33	0.24	3.6	Yo-0	40	0.23	2.6
Ts-6	32	0.25	3.5	Zu-0	38	0.25	3.2
Ts-7	39	0.23	3.2	Zu-1	40	0.25	2.6
Tsu-0	38	0.22	2.9	KSK-1	33	0.23	4.0
Tu-0	36	0.23	3.1	Ber	41	0.27	3.8
Tu-1	38	0.23	2.9	M73235	33	0.23	3.8
Tul-0	39	0.26	3.6	Bu-0	36	0.22	2.9
Ty-0	40	0.26	4.2	Bu-11	38	0.23	3.0
Uk-1	37	0.22	2.7	Do-0	33	0.20	3.4
Uk-2	37	0.24	2.7	Fi-0	35	0.25	2.5
Uk-3	34	0.23	2.8	Fi-1	37	0.21	2.9
Uk-4	34	0.25	2.7	Goc-2	36	0.24	3.1
Van-0	37	0.25	3.0	Gr-3	38	0.22	3.0
Vi-0	41	0.24	2.9	Gr-4	40	0.22	2.7
Wa-1	39	0.21	2.8	Hau-0	39	0.22	3.4
Wc-1	38	0.25	3.4	Hb-0	38	0.24	2.6
Wc-2	38	0.26	3.1	Kl-3	42	0.21	2.7
Wil-1	37	0.21	3.1	Kl-4	42	0.21	2.9
Wil-2	36	0.22	3.4	Mr-0	40	0.23	2.7
Wil-3	38	0.22	3.2				
Wl-0	41	0.26	2.9				
Ws-0	38	0.20	2.7				
Wt-1	37	0.24	3.1				

(continued)

Table 2 (continued)

Accessions	% Oil	Chain length ratios	Desaturation ratios	Accessions	% Oil	Chain length ratios	Desaturation ratios
Wt-2	38	0.24	3.1				
Wt-3	36	0.20	2.7				
Wt-4	36	0.22	3.2				
Wt-5	29	0.22	3.3				
Wu-0	37	0.22	2.7				

chromatographs and provide robust indicators of the results of chain extension activity (e.g. by the product of the *FAE1* locus) and desaturation activity (e.g. by the products of the *FAD2* and *FAD3* loci). The measurements of % oil content, chain length ratio and desaturation ratio for all 360 accessions analysed in the survey screen are shown in Table 2.

2.2. Seed oil content

The oil content of seed samples, expressed on a dry weight basis, was determined for all accessions. The modal oil content was found to be 38%, with a distribution as shown in Fig. 1. Most accessions studied lie within the range 33–43%. Nok-3 and Cvi-0 accession N902 were recorded with the lowest oil content, 28%. The oil content of the duplicate Cvi-0 accession, N1096, was also exceptionally low (31%), in accordance with that found for Cvi-0 accession N902. Other accessions found to have exceptionally low seed oil content were

Col-2 (29%), Wt-5 (29%), Ler-1 (31%), Abd-0 (31%) and Sg-2 (31%). Accessions with the highest seed oil were Enkheim-D (43%), Dijon-M (44%), Ct-1 (44%), Mc-0 (44%) and Ga-0 (45%).

Generally, duplicate accessions had similar seed oil content: Kas-1 (N903 and N1264) contained 38 and 40% respectively, Ag-0 (N901 and N936) contained 35 and 34% respectively, Cvi-0 (N902 and N1096) contained 28 and 31% respectively, Aa-0 (N900 and N934) contained 35 and 32% respectively, Mh-0 (N904 and N1366) contained 41 and 40% respectively, Ms-0 (N905 and N1376) contained 34 and 37% respectively. However, some accessions collected from the same location showed marked differences in oil content. For example, Nok-0, 1, 2 and 3 contained 38, 35, 42 and 28% oil, respectively. This could be due to genetic heterogeneity at the sites of collection of the accessions, but may simply be due to an unreliable datum. For example, replicated analysis of oil content in Nok-3 indicated that oil content in that accession was actually much

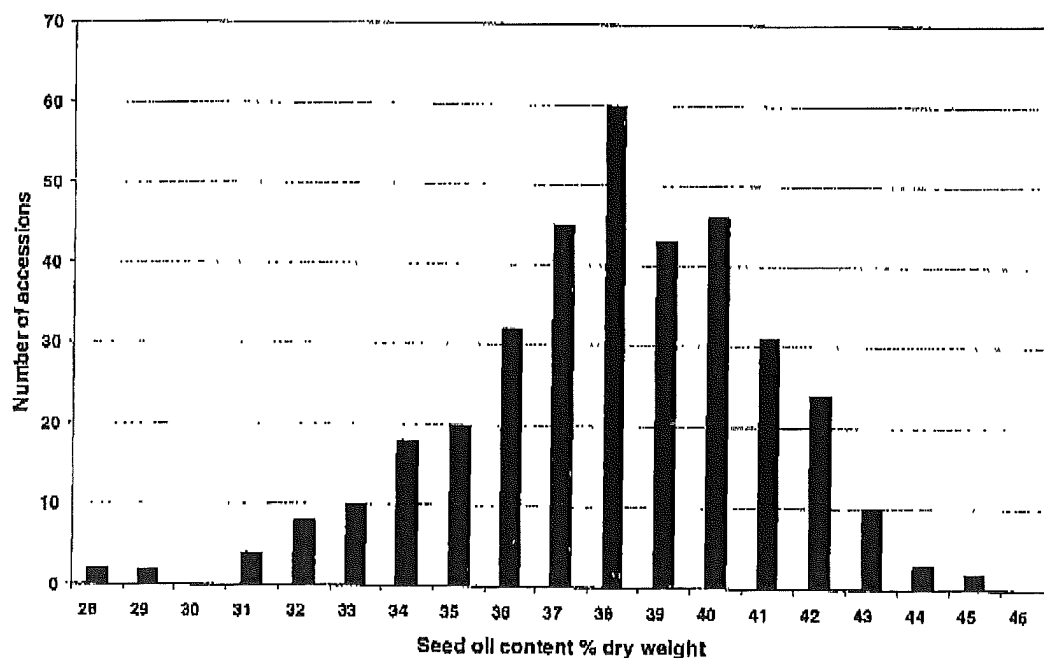


Fig. 1. The distribution of seed oil content in 360 *A. thaliana* accessions.

higher than 28%, so more similar to the others (see Section 2.5).

2.3. Very long chain fatty acid content

The fatty acid methyl ester data generated from all of the accessions were used to calculate fatty acid chain length ratios. The modal value was 0.25, with a distribution as shown in Fig. 2. The majority of the accessions had values between 0.2 and 0.27. The lowest content of VLCFAs was found for four accessions from Tajikistan: Kondara (0.15), Hodja-Obi-Garm (0.16), Shahdara (0.17) and Sorbo (0.17). Other accessions with low content of VLCFAs were Bla-12 (0.18), Cha-0 (0.18), Kas-1 N1264 (0.19) and XXX-0 (0.19). The accessions with the highest proportions of VLCFAs were: Br-0 (0.30), Kl-2 (0.30), Cit-0 (0.31), Gy-0 (0.32), Ga-0 (0.33), Hl-3 (0.33), St-0 (0.33) and Gr-6 (0.35).

Duplicate accessions generally produced similar chain length ratios: Kas-1 (N903 and N1264) yielded values of 0.25 and 0.19 respectively, Ag-0 (N901 and N936) yielded 0.29 and 0.25 respectively, Cvi-0 (N902 and N1096) yielded 0.20 and 0.24 respectively, Aa-0 (N900 and N934) yielded 0.27 and 0.22 respectively, Mh-0 (N904 and N1366) yielded 0.24 and 0.23 respectively, Ms-0 (N905 and N1376) yielded 0.25 and 0.21 respectively.

2.4. Polyunsaturated fatty acid content

The fatty acid methyl ester data generated from all of the accessions were used to calculate fatty acid desaturation ratios. The modal value was 2.9, with a distribution as shown in Fig. 3. The majority of the

accessions had values between 2.4 and 3.8. The lowest content of PUFAs was found for accessions: St-0 (1.8), La-0 (2.0), Mz-0 (2.1) and Dijon G (2.2). The accessions with the highest content of PUFAs were: Kondara (4.2), Ty-0 (4.2), Ost-0 (4.3), Hodja-Obi-Garm (4.3), Shahdara (4.3), Cit-0 (4.2), Col-0 (4.3), Cvi-0 N1096 (4.3) and Cvi-0 N902 (4.4).

Duplicate accessions generally produced similar desaturation ratios: Kas-1 (N903 and N1264) yielded values of 3.6 and 3.4 respectively, Ag-0 (N901 and N936) yielded 3.1 and 2.9 respectively, Cvi-0 (N902 and N1096) yielded 4.4 and 4.3 respectively, Aa-0 (N900 and N934) yielded 3.2 and 2.7 respectively, Mh-0 (N904 and N1366) yielded 2.7 and 3.3 respectively. However Ms-0 (N905 and N1376) yielded ratios of 3.8 and 2.6 respectively.

2.5. A core set of accessions for seed lipid trait diversity

Based on the results of the large scale screening of the 360 accessions, 13 accessions were chosen as candidates for more intensive analysis of seed lipid traits. One of these was the "reference" *A. thaliana* accession, Col-0. The selection of the others was made on the following basis: (1) each accession should have a value near an extreme for at least one of the traits analysed, (2) the set should contain a variety of combinations of extreme and typical trait values and (3) the set should represent a wide diversity of geographic origins. Ten replicates of each of the 13 selected accessions were grown under similar glasshouse conditions to the survey screen, with vernalisation at the seed stage. A further 10 replicates of each accession were grown simultaneously, but without vernalisation. This was done in order to increase the

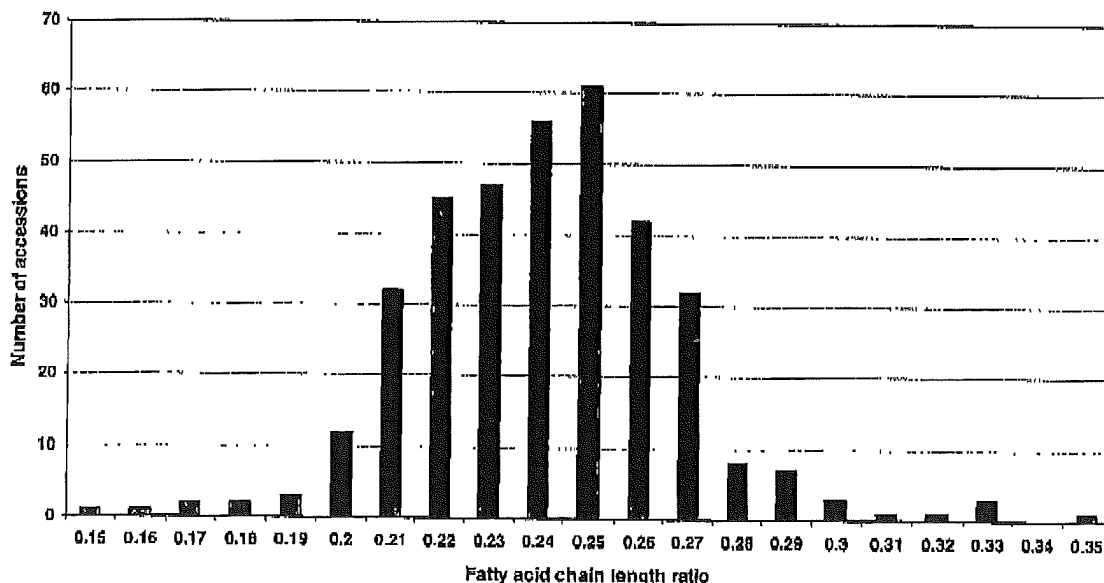


Fig. 2. The distribution of fatty acid chain length in 360 *A. thaliana* accessions.

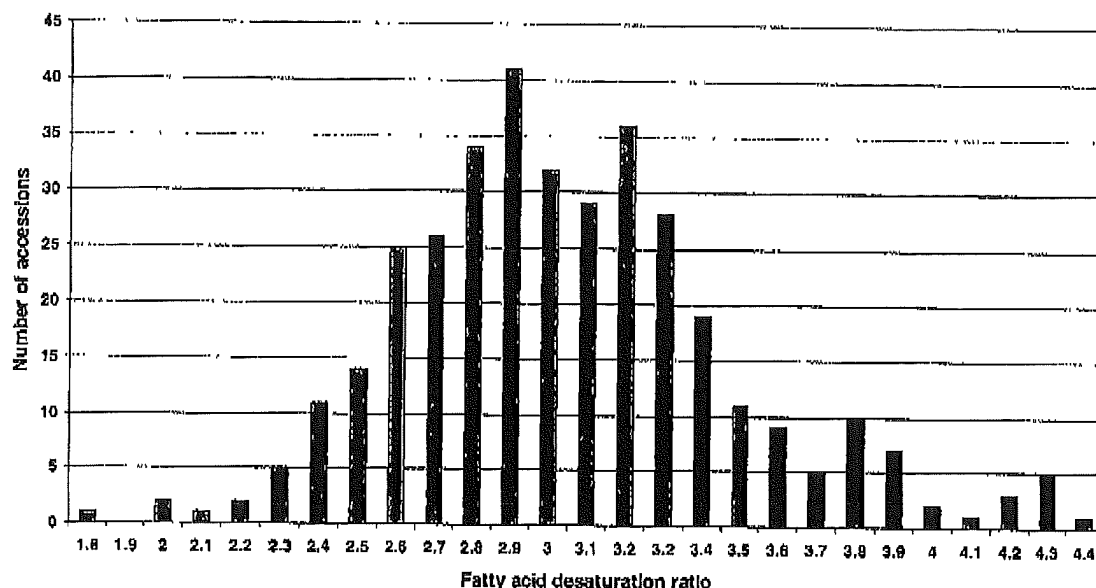


Fig. 3. The distribution of fatty acid desaturation in 360 *A. thaliana* accessions.

number of replicates and to assess any effect of vernalisation on seed lipid traits. The assessment of any effect of vernalisation is important as some *A. thaliana* accessions require the treatment to induce flowering (so we had to apply it to all of the accessions in the survey screen), but it is not usually used with the accessions commonly employed for genetic or biochemical analyses. Mean values for fatty acid composition of the seed oil obtained from vernalised and unvernalsed plants, expressed as a percentage of the nine identifiable fatty acids, are shown in Table 3.

Standard errors are derived from the error mean squares from the two-way analysis of variance with accessions and vernalisation as the main sources of variation. To aid interpretation of the results, the chain length ratio and desaturation ratio, both with and without vernalisation treatment, were calculated for each accession, as previously described. These, along with their respective standard deviations, are shown in Table 4.

Statistical analyses were conducted for oil content, chain length ratio and desaturation ratio using GEN-

Table 3
Proportions of fatty acid methyl esters prepared from total seed lipid

% ^a	16:0		18:0		18:1		18:2		18:3		20:0		20:1		20:2		22:1	
	+ V	-V	+ V	-V	+ V	-V	+ V	-V	+ V	-V	+ V	-V	+ V	-V	+ V	-V	+ V	-V
Ag-0	10.7	10.7	3.0	3.1	10.3	13.4	32.4	32.4	23.8	21.2	1.8	1.8	14.5	14.3	1.8	1.5	1.9	1.6
Br-0	9.7	10.3	3.1	3.1	10.9	13.8	29.3	29.0	26.0	25.6	1.5	1.5	16.0	14.1	1.7	1.4	1.5	1.5
Col-0	10.6	10.4	2.9	2.9	11.3	11.7	35.4	35.4	23.3	22.9	1.3	1.3	12.2	12.3	1.9	1.8	1.0	0.9
Ct-1	9.2	9.6	3.5	3.5	16.6	15.6	33.3	34.1	18.6	19.2	1.6	1.5	15.0	13.4	1.4	1.5	1.2	1.3
Cvi-0	9.8	10.6	2.6	2.5	10.1	10.6	39.7	41.0	19.8	18.8	1.4	1.3	12.7	11.9	2.3	2.1	1.7	1.5
Ga-0	9.3	9.4	3.4	3.5	12.0	12.5	32.4	31.8	23.8	23.7	1.9	1.9	13.6	14.1	1.8	1.6	1.7	1.7
Gy-0	10.5	10.5	3.1	3.0	8.7	10.1	30.3	30.0	26.0	25.3	1.9	1.9	15.2	14.9	2.1	1.8	2.0	2.3
Kond	9.0	8.4	2.4	2.4	10.7	11.7	40.6	42.5	23.8	22.3	1.0	0.9	9.4	9.4	1.7	1.7	1.3	1.0
Mz-0	9.9	11.1	2.6	2.5	10.7	10.2	35.5	37.8	22.2	21.8	1.3	1.2	14.2	11.9	2.1	1.9	1.7	1.5
Nok-3	10.1	10.3	3.3	3.3	13.8	14.6	30.6	32.0	23.2	21.6	1.7	1.6	13.9	14.0	1.5	1.3	1.5	1.3
Sorbo	8.4	8.1	2.1	2.1	13.1	14.8	38.4	39.8	24.1	22.7	0.8	0.8	10.0	9.5	1.6	1.4	1.3	1.2
Ts-5	11.3	12.1	3.1	2.9	9.7	11.3	31.4	33.6	25.0	23.5	1.6	1.4	14.5	12.6	1.8	1.5	1.8	1.2
Wt-5	11.5	11.5	2.9	2.9	11.0	11.5	28.9	29.6	26.8	25.2	1.5	1.5	14.2	14.2	1.6	1.5	1.8	1.7
S.E.	0.14		0.04		0.22		0.36		0.28		0.03		0.31		0.06		0.09	

+ V: vernalised plants, -V: unvernalsed plants.

^a Percentage of total integrated peaks on chromatographs identified as fatty acids; mean of 10 values.

Table 4
Seed lipid traits of a core set of *Arabidopsis thaliana* accessions from survey and replicated experiments

Accession	Origin	% Oil					Chain length ratio					Desaturation ratio				
		S	+V	S.D.	-V	S.D.	S	+V	S.D.	-V	S.D.	S	+V	S.D.	-V	S.D.
Ag-0 (N936)	France	34	40.8	1.81	39.6	2.58	0.25	0.227	0.013	0.219	0.014	2.9	4.23	0.23	3.29	0.43
Br-0	Czechoslovakia	40	39.1	1.24	37.9	1.61	0.30	0.241	0.019	0.209	0.006	3.1	3.96	0.01	3.25	0.20
Col-0	Uncertain	36	39.7	1.49	41.9	1.57	0.24	0.173	0.008	0.174	0.008	4.3	4.14	0.16	4.01	0.11
Ct-1	Italy	44	44.9	1.13	46.0	1.31	0.23	0.219	0.011	0.198	0.011	2.5	2.58	0.09	2.80	0.12
Cvi-0 (N902)	Cape Verdi Is.	28	34.6	3.46	34.9	1.65	0.20	0.193	0.015	0.176	0.008	4.4	4.69	0.23	4.59	0.15
Ga-0	Germany	45	42.2	2.34	44.2	2.17	0.33	0.211	0.040	0.217	0.026	2.7	3.68	0.23	3.47	0.20
Gy-0	France	37	38.1	1.65	39.7	1.31	0.32	0.242	0.009	0.241	0.005	3.8	4.76	0.31	4.22	0.16
Kondara	Tajikistan	35	38.5	1.20	38.3	1.75	0.15	0.135	0.013	0.130	0.012	4.2	4.90	0.18	4.63	0.38
Mz-0	Germany	39	40.4	1.60	40.8	1.35	0.25	0.211	0.038	0.174	0.012	2.1	4.36	0.13	4.72	0.42
Nok-3	Netherlands	28	42.3	1.16	42.0	1.23	0.24	0.210	0.008	0.206	0.015	3.1	3.16	0.22	3.00	0.16
Ts-5	Spain	33	38.5	1.08	37.6	1.48	0.24	0.221	0.023	0.180	0.026	3.6	4.44	0.20	4.04	0.31
Sorbo	Tajikistan	33	38.7	2.80	37.9	1.31	0.17	0.141	0.005	0.131	0.005	3.3	4.12	0.28	3.72	0.08
Wt-5	Germany	29	40.1	1.37	39.2	1.12	0.22	0.214	0.007	0.214	0.011	3.3	4.00	0.10	3.80	0.15

S: Measurement from survey experiment, +V: mean from second analysis (vernalised plants), -V: mean from second analysis (unvernalised plants).

STAT (Payne, 2000). The results of the analysis of variance are summarised in Table 5. All three measured traits showed highly significant among line variation ($P < 0.001$). Additionally, desaturation ratio and chain length ratio showed highly significant interactions with vernalisation ($P < 0.001$). There was, however, no significant effect of vernalisation on oil content. The relative biological importance of these effects was estimated by calculating the components of variation for each treatment (σ_{line} , $\sigma_{\text{vernalisation}}$ and $\sigma_{\text{line,vernalisation}}$). The among line variation accounted for the largest part of the total variation: 67.6% for chain length ratio, 66.2% for oil content and 71.4% for the desaturation ratios. The variation accounted for by vernalisation treatment (6.8 and 6.3% for chain length ratio and desaturation ratio, respectively) was much smaller, confirming the predominant effect of differing accessions.

Although absolute values varied, the trends in the data from the replicated experiment largely confirmed the results of the survey screen. For example, the highest oil accessions further analysed, Ct-1 and Ga-0, were confirmed as containing the highest proportions of oil and one of the lowest oil accession, Cvi-0, was con-

firmed as containing the lowest proportion of oil. However, Nok-3, which also had an exceptionally low oil content in the survey screen, was found to have a typical oil content in the replicated experiment. The accession with the lowest proportion of VLCFAs in the survey screen, Kondara, was confirmed as the lowest and two of the three with the highest VLCFA content, Br-0 and Gy-0 were confirmed as containing the highest. There was less consistency in the results for PUFA content. The accession with the lowest content in the replicated experiment, Ct-1, was only the second lowest in the survey screen of those re-analysed and the accession with the highest PUFA content in the replicated experiment, Kondara, was only the third highest in the survey screen of those re-analysed. The differences observed between the survey screen and the replicated experiment are probably due to a combination of environmental differences between the seasons in which the plants were grown and our selection of accessions with extreme values in the survey screen, which would have been those most likely to be aberrant. It is also possible that competition effects resulting from the growth of four plants per pot in the survey screen may have affected the results.

Table 5
Analysis of variance for seed lipid traits in replicated experiment

	Mean squares from ANOVA			
	Line (d.f. = 12)	Vernalisation (d.f. = 1)	Line × vernalisation (d.f. = 12)	Error mean square (d.f. = 234)
Chain length	0.0226***	0.0154***	0.0013***	0.00031
Desaturation ratio	7.520***	4.593***	0.610***	0.0517
Oil content (% dry wt.)	142.165***	1.334**	7.60**	2.97
<i>Components of variation (%)</i>				
Chain length	67.6	6.8	6.3	19.3
Desaturation ratio	71.4	6.3	11.6	10.7
Oil content (% dry wt.)	66.2	0.0	4.6	29.2

** $P < 0.01$. *** $P < 0.001$.

2.6. Regions of the *A. thaliana* genome associated with control of the traits

Having identified variation between accessions, we aimed to begin the characterisation of the underlying genetics. Two accessions with divergent seed lipid characteristics were crossed: Kondara (low chain length ratio, high desaturation ratio, medium oil content) and Br-0 (high chain length ratio, medium desaturation ratio and medium oil content). An F_2 population of 59 plants was produced and, after vernalisation, was grown in standard glasshouse conditions. The oil content and fatty acid composition of the seeds produced by individual plants were measured. The results are summarised in Fig. 4 for oil content, Fig. 5 for chain length ratio and Fig. 6 for desaturation ratio. Segregation could be identified for all three traits, indicating a genetic basis for the variation. It should be noted that segregation for seed oil content was observed, as well as for chain length and desaturation ratios, despite the parents of the cross differing little for this trait.

The control of quantitative traits such as seed oil content and fatty acid composition is expected to be complex, i.e. involving multiple genes. In order to search for candidate genes with major effects, we conducted an analysis of association between the traits and the genotype of F_2 plants. Early in the growth of the F_2 population, one leaf was removed from each plant and assayed for 18 polymorphic Simple Sequence Repeat

(SSR) markers dispersed throughout the genome (nga63, AthS0392, T27K12-Sp6, AthGENEA, AthATPASE, nga1145, nga1126, nga168, AthUBIQUE, nga172, AthGAPAb, PUR5a, AthDET1, nga1107, nga225, nga151, nga139, nga129; (http://genome.salk.edu/SSLP_info). Marker-trait associations were identified using GenStat (Payne, 2000). Significant associations were identified for oil content, chain length ratio and desaturation ratio, as shown in Table 6. Although the markers involved are not very tightly linked, these results are consistent with *FAE1* (which, like marker AthDET1, is on chromosome 4) being a candidate for underlying some of the variation in chain length ratio, and *FAD3* (which, like marker AthUBIQUE, is on chromosome 2) being a candidate for underlying some of the variation in desaturation ratio. The allele associated with higher chain length ratio comes from Br-0 and the allele associated with higher desaturation ratio comes from Kondara. We can identify no clear candidate genes underlying variation in oil content.

3. Discussion

All 360 accessions of *A. thaliana* that were available from the *Arabidopsis* resource centres and could be grown to maturity were screened for natural variation in seed lipid traits. Although the analysis conducted was,

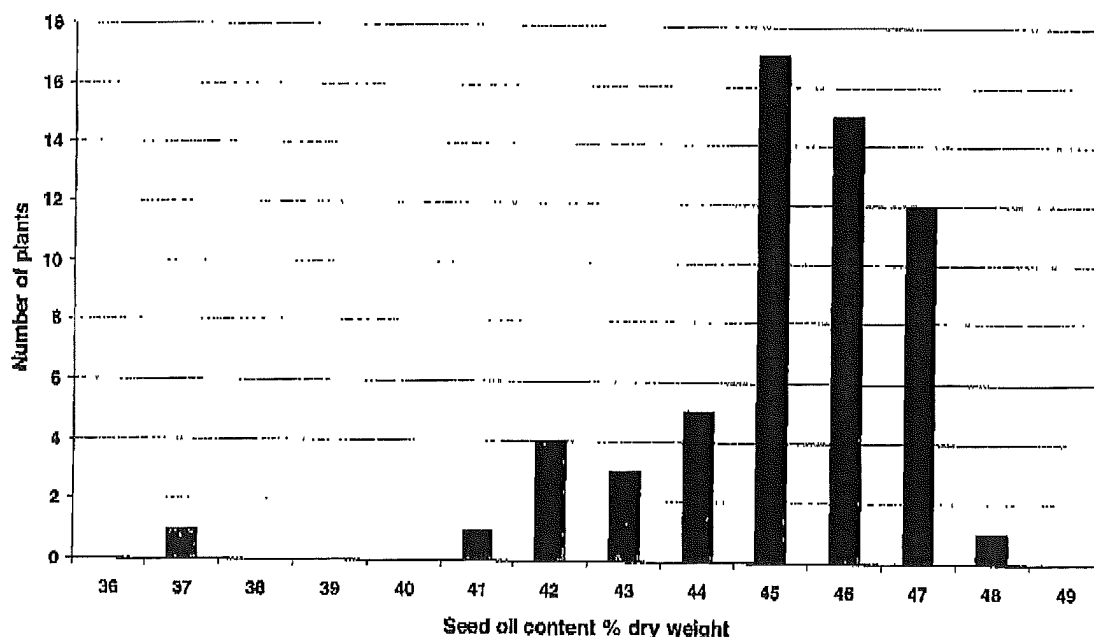


Fig. 4. The segregation of seed oil content in an F_2 population derived from a cross between accessions Kondara and Br-0.

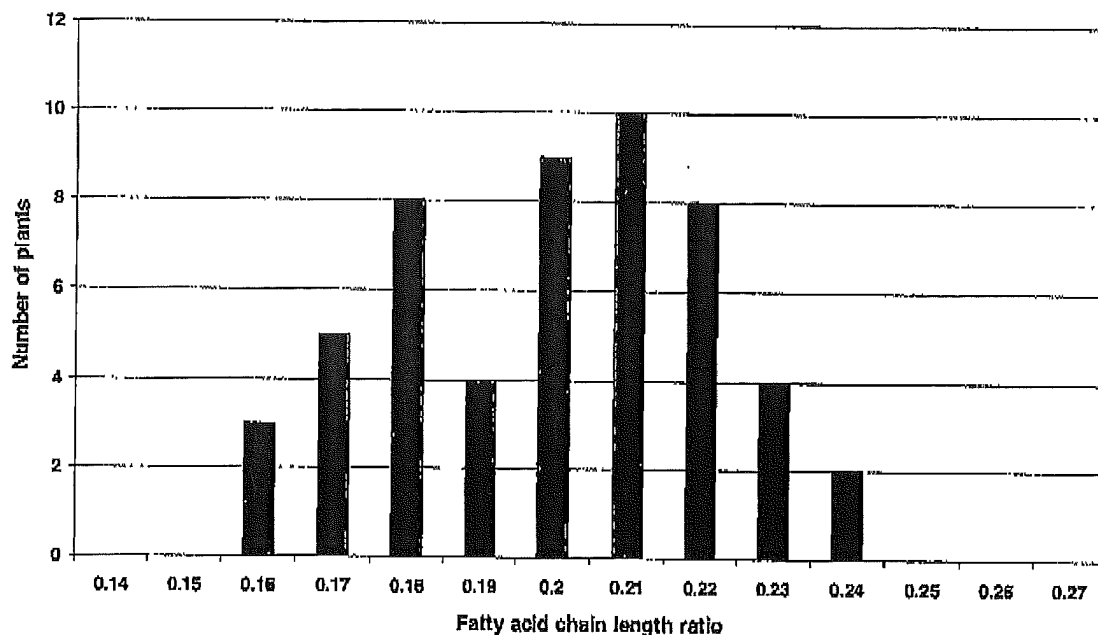


Fig. 5. The segregation of fatty acid chain length ratio in an F_2 population derived from a cross between accessions Kondara and Br-0.

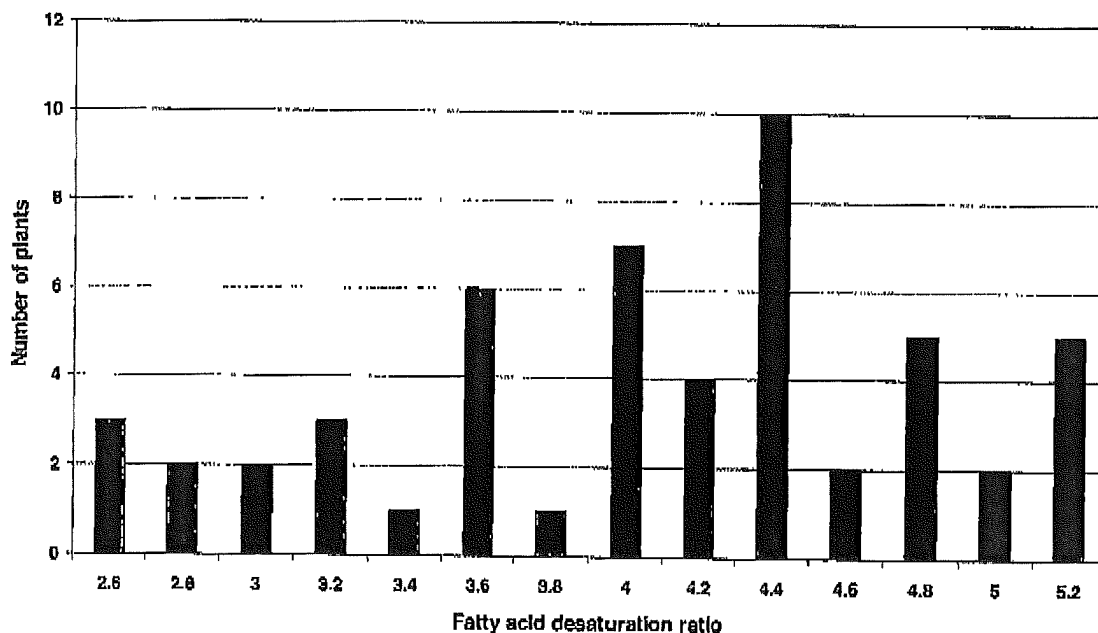


Fig. 6. The segregation of fatty acid desaturation ratio in an F_2 population derived from a cross between accessions Kondara and Br-0.

for logistical reasons, a survey with pooling of plants rather than a fully replicated analysis, the results have been shown to be broadly representative of the variation available. Specific lines producing seeds with reproducibly extreme content of oil, VLCFA and PUFA content have been identified.

Reproducible variation of VLCFA content of seed was confirmed for several accessions in a replicated analysis. As shown in Table 4, the content of VLCFAs in seeds, expressed as the ratio of proportions of long chain (20 and 22 carbon) to medium chain (16 and 18 carbon) fatty acids, varied from 0.130 to 0.242. This

Table 6
Marker-trait associations detected

Trait	F pr	Marker	Primers for marker	Nearest gene
Oil content	0.011**	AthUBIQUE	AGGCAAATGTCCATTTCATTG ACGACATGGCAGATTCTCC	At2g46030
Oil content	0.031*	ngal72	AGCTGCTTCCTATAGCGTCC CATCCGAATGCCATTGTTT	At3g03330
Chain length	0.047*	AthDET1	TTCAAACACCAATATCAGGCG GGTGAAAATGGAGGAGACGA	At4g10170
Desaturation	0.047*	AthUBIQUE	AGGCAAATGTCCATTTCATTG ACGACATGGCAGATTCTCC	At2g46030

* $P < 0.05$; ** $P < 0.01$.

corresponds to a range of 13.0–21.2% of total identified fatty acids, as shown in Table 3. The results of a survey of a subset of accessions for VLCFA content in seeds have previously been published (Millar and Kunst, 1999). As in that investigation, we also found no evidence of unusual fatty acids, although there were some minor GC peaks, present in all samples, which could not be assigned as fatty acids. The four accessions we identified in our survey with the lowest VLCFA content (Kondara, Hodja-Obi-Garm, Shahdara and Sorbo) were also identified as having exceptionally low VLCFA content in this previous study. Of the highest VLCFA content accessions (Br-0, Kl-2, Cit-0, Gy-0, Ga-0, Hl-3, St-0 and Gr-6), only two had been analysed before: Gy-0, which had also been found to contain an exceptionally high proportion of VLCFAs and St-0, which had been reported as containing only a moderate amount. Therefore our results are in good agreement with, and considerably extend, previous studies of VLCFA content of seeds of *A. thaliana* accessions. The key biochemical step in the synthesis of VLCFAs in the endoplasmic reticulum of cells of developing *A. thaliana* embryos is catalysed by the product of the *FAEI* locus, the transcription level of which has a quantitative effect on the proportions of VLCFAs in storage oil (Millar and Kunst, 1997). The natural variation for VLCFA content, in a population derived from a cross between accessions Kondara and Br-0, showed an association with the region of the genome containing *FAEI*, consistent with a role for *FAEI* in the regulation of VLCFA content.

Our data represent the first reported extensive survey of natural variation for oil and PUFA content in seeds of *A. thaliana* accessions. Both traits vary widely. As shown in Table 2, reproducible oil contents, expressed as a proportion of seed dry weight, varied from 34.6 to 46.0%. Reproducible PUFA contents, expressed as the ratio of proportions of polyunsaturated 18 carbon to saturated and monounsaturated 18 carbon fatty acids, varied from 2.58 to 4.90. This corresponds to a range of 53.3–66.1% of total identified fatty acids, as shown in

Table 3. The biosynthesis of PUFAs for storage in seeds is dependent upon the successive action in the endoplasmic reticulum of the enzymes encoded at two loci: *FAD2*, which encodes the oleate desaturase, synthesising linoleic acid, and *FAD3*, which encodes the linoleate desaturase, synthesising linolenic acid (Okuley et al., 1994; Arondel et al., 1992). Mutations in *FAD2* are recessive, suggesting that this locus does not have a quantitative effect on PUFA synthesis, but mutations in *FAD3* are semi-dominant and over-expression of *FAD3* quantitatively reduces linoleic acid content and increases linolenic acid content (Shah et al., 1997). Thus *FAD3* is a good candidate for natural allelic variation contributing to quantitative variation of PUFA content. The natural variation for PUFA content, in a population derived from a cross between accessions Kondara and Br-0, showed an association with the region of the genome containing *FAD3*, consistent with the hypothesis that *FAD3* is involved in the quantitative regulation of PUFA content.

The vernalisation treatment of plants very early in their life cycle would not be expected to have an effect on the lipid composition of the seeds they produce. We saw no evidence for any effect on oil content. However, there is a significant interaction between vernalisation treatment and both PUFA and VLCFA content. This is most striking for PUFA content, where 10 of the 12 accessions produced seeds with a higher desaturation ratio when they had been vernalised compared to when they had not. In two cases (Ag-0 and Br-0) the differences in the means of the desaturation ratios were in excess of two standard deviations. Some accessions may, therefore, show an environmental interaction whereby temperature conditions early in development could affect the extent of desaturation and chain elongation of the fatty acids in their seeds at maturity. A detailed analysis of this will require further investigations, but vernalisation, even at the seed stage, should clearly be taken into account when analysing the fatty acid composition of seeds of *A. thaliana*.

We have identified a core set of accessions that contain a wide range of reproducible phenotypic variation for seed lipid traits. Further genetic analysis can now be conducted using these accessions to understand the genetics underlying regulation and environmental interaction of seed lipid traits and to determine the balance of contributions from allelic variation of the major components of the biosynthetic pathway and other, regulatory, genes.

4. Experimental

4.1. Plant material

A. thaliana accessions were grown on two separate occasions. In the first experiment, seeds for 364 different accessions were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Of these, 360 could be grown and produced sufficient seed for analysis, as listed in Table 1. The first 43 accessions listed, Aa-0 to Wci-1 are non-*Arabidopsis* Information Service (AIS) stocks. The remaining 317 accessions are AIS stocks. Where there are accessions with the same name in each collection, we refer to these as duplicate accessions and cite the respective NASC stock numbers. All other accessions are uniquely identified by their accession name. Approximately 20 seeds from each accession were sown out onto *Arabidopsis* soil mix: Levington's M2 compost with 4 mm grit (8:1) and Intercept, which protects against aphids. Seeds were then transferred directly to a 5 °C chamber, with 8-h photoperiod for 6 weeks to vernalise. Most accessions germinated during this time, though there were a few which germinated only after the transfer at the end of that period to a glasshouse at 18 °C day/15 °C night, 16 h photoperiod with supplementary sodium lamps. When the first true leaves developed, four seedlings per accession were pricked out to *Arabidopsis* soil mix in 7 cm pots. When the oldest siliques on the plant were starting to brown, the plants were dried off. Bulk seed was collected from the four plants in each pot for the seed lipid analysis. In the second experiment, 12 selected accessions, as listed in Table 2 were grown with replicates. Ten plants were grown for each accession following vernalisation at the seed stage for 6 weeks, as described above. Seeds for 10 more plants per accession were stratified at 5 °C for 5 days. Plants were transferred to glasshouse conditions as described for the main screen, except that plants were grown individually in 7 cm pots and their positions in the glasshouse were randomised.

4.2. Analysis of seed oil

To investigate the total oil content in each accession seeds free of plant debris were analysed using a nuclear

magnetic resonance machine Oxford QP20+. Fifty mg samples from the bulked seed of four plants were prepared and analysed per accession. The oil content of the seeds was expressed as % dry weight, as calculated by the instrument.

For fatty-acid analysis, 50 mg seed samples were boiled in Methanolic HCl according to the method of James and Dooner (1990). Samples were extracted in hexane. Gas-liquid chromatography was performed on a Perkin Elmer Autosystem gas chromatograph, using a 50 m Cp-Sil88 capillary column. From each sample, 1 microlitre was injected using an auto-sampler with a split ratio of 1 in 60. The injection port temperature was 270 °C, the oven temperature was 190 °C and detector temperature was 270 °C. Helium was used as the carrier gas at a pressure of 24.4 psi. For peak identification, the oil reference standard for low erucic rapeseed (Sigma) was run with the samples. The conditions allowed the detection of the fatty acid methyl esters of: 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 22:1. To provide simple metrics for comparison of the accessions, we calculated ratios for carbon chain length and for desaturation using the quantification from GC chromatograms. The chain length ratio is the sum of areas under peaks for all 20 and 22 carbon fatty acids divided by the sum of areas under peaks for all 16 and 18 carbon fatty acids. The desaturation ratio is the sum of areas under peaks for polyunsaturated 18 carbon fatty acids divided by the sum of areas under peaks for saturated and mono-unsaturated 18 carbon fatty acids.

4.3. Analysis of data

Analysis of variance of the data from experiment two was used to estimate levels of significance among lines, between treatments and of their interactions. Additionally, components of variation were calculated to estimate the relative biological importance of these sources of variation. Analyses were carried out using the statistical package GENSTAT (Payne, 2000).

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References

- Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C.J., Koorneef, M., 1999. Natural allelic variation at seed size loci in relation to other life history traits of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 96, 4710–4717.

- Arabidopsis Genome Initiative, 2000. Analysis of the genome of the flowering plant *Arabidopsis thaliana*. *Nature* 408, 796–815.
- Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H.M., Somerville, C.R., 1992. Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. *Science* 258, 1353–1355.
- Cavell, A.C., Lydiate, D.C., Parkin, I.A.P., Dean, C., Trick, M., 1998. Collinearity between a 30-centimorgan segment of *Arabidopsis thaliana* chromosome 4 and duplicated regions within the *Brassica napus* genome. *Genome* 41, 433–440.
- James Jr., D.W., Dooner, H.K., 1990. Isolation of EMS-induced mutants in *Arabidopsis* altered in seed fatty acid composition. *Theor. Appl. Genet.* 80, 241–245.
- James, D.W., Lim, E., Keller, J., 1995. Directed tagging of the *Arabidopsis* fatty-acid elongation-1 (FAE1) gene with the maize transposon Activator. *Plant Cell* 7, 309–319.
- Katavic, V., Reed, D.W., Taylor, D.C., Giblin, E.M., Burton, D.L., Zou, J., MacKenzie, S.L., Covello, P.S., Kunst, L., 1995. Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. *Plant Physiol* 108, 399–409.
- Koorneef, M., Alonso-Blanco, C., Peters, A.J.M., Soppe, W., 1998. Genetic control of flowering time in *Arabidopsis*. *Annu. Rev. Plant Physiol* 49, 345–370.
- Krannitz, P.G., Aarssen, L.W., Dow, J.M., 1991. The effect of genetically based differences in seed size on seedling survival in *Arabidopsis thaliana* (Brassicaceae). *Am. J. Bot* 78, 446–450.
- Lemieux, B., Miquel, M., Somerville, C., Browse, J., 1990. Mutants of *Arabidopsis* with alterations in seed lipid fatty acid composition. *Theor. Appl. Genet.* 80, 234–240.
- Millar, A.A., Kunst, L., 1997. Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *Plant J.* 12, 121–131.
- Millar, A.A., Kunst, L., 1999. The natural genetic variation of the fatty-acyl composition of seed oils in different ecotypes of *Arabidopsis thaliana*. *Phytochemistry* 52, 1029–1033.
- Mitchell-Olds, T., 1996. Genetic constraints on life history evolution: quantitative trait loci influencing growth and flowering in *Arabidopsis thaliana*. *Evolution* 50, 140–145.
- Mithen, R.F., Clarke, J., Lister, C., Dean, C., 1995. Genetics of aliphatic glucosinolates. III. Side chain modifications in *Arabidopsis thaliana*. *Heredity* 74, 210–215.
- Ohlrogge, J., Browse, J., 1995. Lipid biosynthesis. *Plant Cell* 7, 957–970.
- Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., Browse, J., 1994. *Arabidopsis* FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *Plant Cell* 6, 147–158.
- Pathak, M.K., Ghosh, D., Maji, M.K., Ghosh, S., 1994. Oil content and fatty acid composition of seeds of various ecotypes of *Arabidopsis thaliana*: a search for new useful genetic variants. *Current Science* 67, 470–472.
- Payne, R.W., 2000. *GenStat: The Guide to GenStat*, fourth ed. VSN International, Oxford.
- Poirier, Y., Ventre, G., Caldelari, D., 1999. Increased flow of fatty acids towards beta-oxidation in developing seeds of *Arabidopsis* deficient in diacylglycerol acyltransferase activity of synthesizing medium-chain-length fatty acids. *Plant Physiol.* 121, 1359–1366.
- Rushotte, A.M., Jenks, A.M., Nyugen, T., Feldman, K.A., 1997. Epicuticular wax variation in ecotypes of *Arabidopsis thaliana*. *Phytochemistry* 45, 251–255.
- Shah, S., Xin, Z., Browse, J., 1997. Overexpression of the FAD3 desaturase gene in a mutant of *Arabidopsis*. *Plant Physiol* 114, 1533–1539.